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14. ABSTRACT During development, neurons become dependent on target-derived neurotrophins for survival and maintenance of differentiated functions. Failed or inappropriate target interactions in vivo, or withdrawal of neurotrophins in vitro, lead to a characteristic sequence of molecular cell death events termed "apoptosis". The purpose of the proposed research is to examine the roles of the <i>Nf1</i> gene product, neurofibromin, in modulating the apoptotic response to neurotrophin withdrawal, as well as the survival response to depolarization. Over the past year, comparisons of the responses of <i>Nf1</i> <sup>-/-</sup> (haploinsufficient) and <i>Nf1</i> <sup>+/+</sup> mouse sensory and sympathetic neurons, isolated from several different embryonic stages, to neurotrophin- and activity-mediated survival signaling were completed. <i>Nf1</i> haploinsufficient neurons are more sensitive to suboptimal neurotrophin doses and depolarization, and survive longer after nerve growth factor is withdrawn. In addition, the effects of NGF exposure on the acquisition of neurotrophin dependence by <i>Nf1</i> <sup>-/-</sup> and <i>Nf1</i> <sup>+/+</sup> sensory and sympathetic neurons was examined, using cells isolated from early (E12) mouse embryos. Although neurofibromin-deficient neurons are sensitive to certain apoptotic stimuli (e.g. C2-ceramide), they are resistant to other signals for programmed cell death. The jun kinase/ c-jun pathway may be involved in this resistance to apoptosis.					
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## INTRODUCTION

During development, peripheral neurons become dependent on target-derived neurotrophins for survival and maintenance of differentiated functions (Davies, 2003). Failed or inappropriate target interactions *in vivo*, or withdrawal of neurotrophins *in vitro*, lead to a characteristic sequence of molecular cell death events termed "apoptosis". The purpose of the proposed research is to examine the roles of the *Nf1* gene product, neurofibromin, in modulating the apoptotic response to neurotrophin withdrawal, as well as the survival response to depolarization. We have shown that many sensory, and almost all sympathetic neurons isolated from *Nf1*<sup>-/-</sup> mouse embryos survive in the absence of neurotrophins (Vogel et al., 1995). With the addition of experiments utilizing neonatal DRG and SCG neurons (Tasks 1 and 3), and continued examination of c-jun and jun-kinase signaling following NGF withdrawal and ceramide exposure (Tasks 4 and 7), we intend to submit two manuscripts within the next year.

## BODY

### **Task 1. Characterize responses of *Nf1*<sup>+/+</sup> and *Nf1*<sup>+/-</sup> DRG and SCG neurons to activity-mediated survival signaling *in vitro*.**

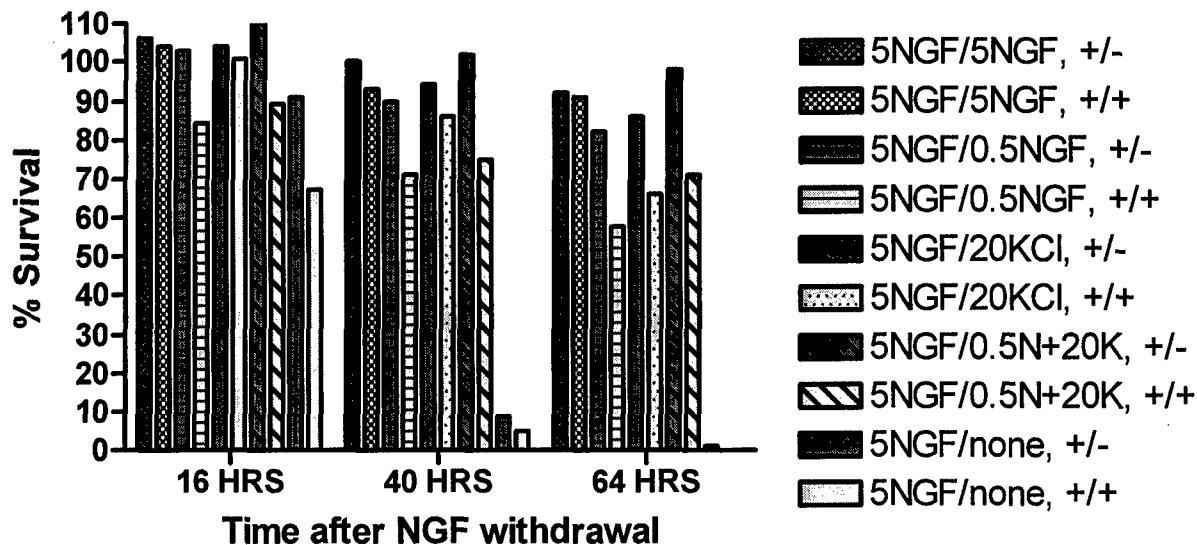
Levels of intracellular calcium and potassium are known to influence apoptosis, particularly in postmitotic neurons (reviewed by Yu et al., 2001). Our hypothesis was that neurons with lower amounts of neurofibromin (*Nf1*<sup>+/-</sup>) would be more sensitive to the survival-promoting effects of high potassium than would wild-type (*Nf1*<sup>+/+</sup>) neurons, due to higher levels of activated Ras. In a previous progress report, we demonstrated significant, reproducible differences in both apoptosis rate and survival response to depolarizing levels of potassium, between dorsal root ganglion (DRG) neurons isolated from *Nf1*<sup>+/+</sup> and *Nf1*<sup>+/-</sup> mouse embryos at E15 and E17. Over the past year, we have extended this result using sympathetic superior cervical ganglion (SCG) neurons isolated from *Nf1*<sup>+/+</sup> and *Nf1*<sup>+/-</sup> mouse embryos at E15, E17, and E18. Our results with E18 DRG and SCG neurons indicate that the differential response of *Nf1*<sup>+/+</sup> and *Nf1*<sup>+/-</sup> neurons may become more significant in late embryonic, and possibly postnatal, development; therefore, we propose to complete this series of experiments using neonatal DRG and SCG neurons. The experiments in Tasks 1 and 3 will be combined for a manuscript.

### **Task 3. Characterize synergy of neurotrophin- and activity-mediated survival signaling in *Nf1*<sup>+/+</sup> and *Nf1*<sup>+/-</sup> DRG and SCG neurons.**

DRG Neurons. To date, we have focused on the survival responses of E17/E18 DRG neurons to depolarizing KCl and suboptimal NGF concentrations, following NGF withdrawal at 72-96 hours *in vitro*. At this stage, DRG neurons are less sensitive to NGF withdrawal, but significant differences between *Nf1*<sup>+/+</sup> and *Nf1*<sup>+/-</sup> cells are apparent. The increased sensitivity of *Nf1*<sup>+/-</sup> neurons to low doses of NGF is consistent with our previous data utilizing *exon23a*<sup>-/-</sup> DRG, trigeminal, and SCG neurons (Brannan and Vogel, unpublished data). In the past year, we have completed these experiments with E15 *Nf1*<sup>+/+</sup> and <sup>+/-</sup> DRG neurons, and plan to characterize synergy in neonatal DRG neurons within the next few months.

SCG Neurons. Figure 1 shows that E17 SCG sympathetic neurons isolated from *Nf1*<sup>+/-</sup> embryos are more sensitive to depolarizing KCl and to low doses of NGF, when compared to neurons isolated from *Nf1*<sup>+/+</sup> littermates. However, it is difficult to say at this point whether there is synergy between neurotrophin and activity-mediated signaling, or if there is simply an additive effect. We have completed experiments with E15, E17, and E18 SCG neurons, and plan to characterize synergy in neonatal SCG neurons within the next few months. The combined data from Tasks 1 and 3, along with the inhibitor studies from Task 2, should constitute a full-length manuscript, to be submitted to *Journal of Neuroscience* once the experiments with neonatal SCG and DRG neurons are complete.

**Figure 1. Survival Signaling  
E17 SCG Neurons**



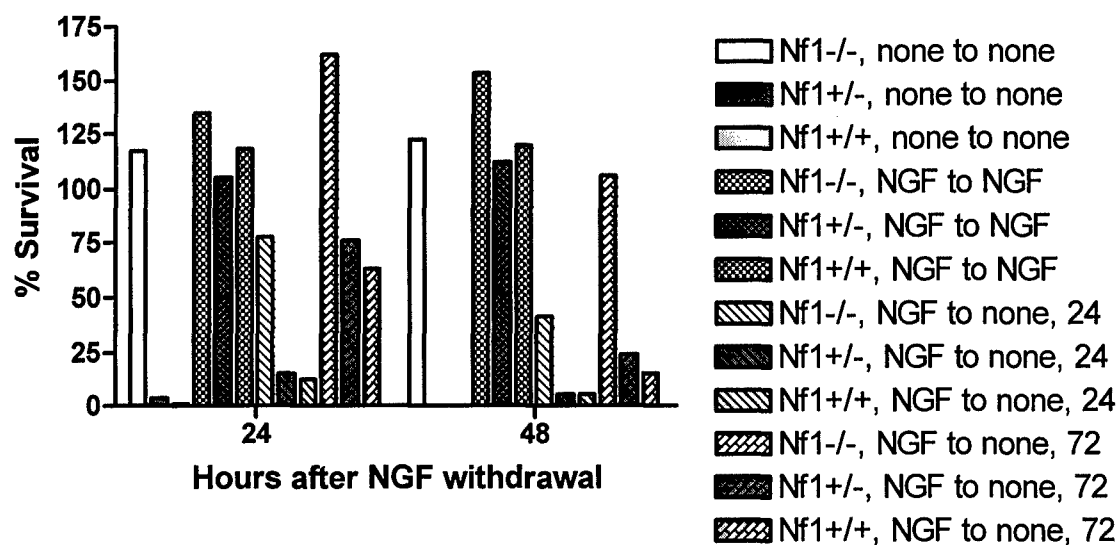
**Figure 1.** Dissociated cultures of E17 SCG neurons were maintained in 5 ng/ml NGF in serum-free (Neurobasal) medium for 96 hours. NGF was withdrawn from all cultures by repeated gentle washes, and replaced with suboptimal doses of NGF (0.5 ng/ml) and/or KCl (20 mM). Control cultures received 5 ng/ml NGF, or no factors. Survival is expressed as a percentage of the initial cohort of neurons counted for each culture at 24 hours.

**Task 4. Characterize the role of neurofibromin in mediating neuronal apoptosis following neurotrophin withdrawal.**

**DRG Neurons.** During the first year of funding, we characterized the response of E12.5 *Nf1*<sup>-/-</sup> DRG neurons to NGF withdrawal and KCl rescue. Based on our previous results (Vogel et al., 1995), we predicted that *Nf1*<sup>-/-</sup> DRG neurons would not undergo apoptosis following NGF withdrawal, whereas neurons isolated from *Nf1*<sup>+/+</sup> and +/- littermates should die 24-48 hours after NGF is removed. To our surprise, we found that approximately 50% of E12.5, and 60% of E13.0, *Nf1* mutant DRG neurons undergo apoptosis within 24-48 hours of NGF withdrawal. In contrast to the loss of many *Nf1*<sup>-/-</sup> DRG neurons following NGF removal, we did not observe apoptosis in sister cultures of neurofibromin-deficient neurons that had never been exposed to NGF *in vitro* (**Figure 2**). For E13 *Nf1*<sup>-/-</sup> DRG neurons deprived of NGF, depolarizing KCl effects a complete rescue, and a suboptimal dose of NGF (0.5ng/ml) rescues over 80% of the cells. In contrast, fewer than 40% of E13 *Nf1*<sup>+/+</sup> DRG neurons can be rescued by activity-mediated signaling, and over 90% undergo apoptosis in the presence of the low dose of NGF (data provided in previous report).

Based on the above, and other unpublished results, we propose that target contact, and concomitant exposure to neurotrophins, initiates the development of neurotrophin dependence in peripheral neurons, even if they lack neurofibromin. Environmental cues encountered by growing axons *en route* to the target undoubtedly influence acquisition of neurotrophin dependence; in cultures of ganglia isolated from developing embryos, some neurons may have extended axons towards or even contacted the peripheral target, whereas others have not yet developed axons. To begin to address these possibilities, we have initiated experiments to determine whether the degree of prior NGF exposure influences the rate or extent of apoptosis among *Nf1*<sup>-/-</sup> neurons, following withdrawal (**Figure 2**).

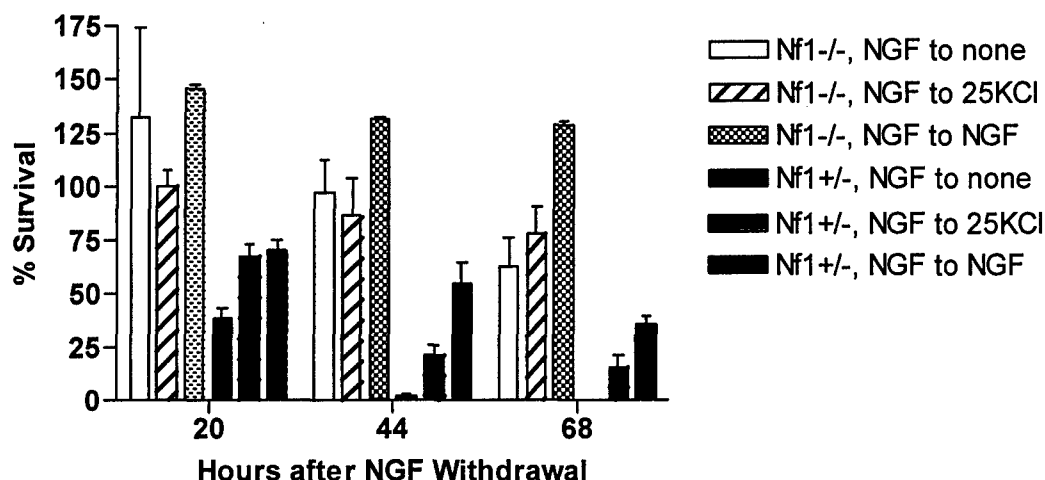
**Figure 2. NGF Withdrawal  
Survival of E12 DRG Neurons**



**Figure 2.** Dissociated cultures of E12 DRG neurons were maintained in 5ng/ml NGF in serum-free medium for 24 or 72 hours, at which point NGF was withdrawn. Survival is expressed as a percentage of the initial cohort of neurons counted for each culture at 24 hours.

**SCG Neurons.** We reported previously that SCG neurons isolated from *Nf1*<sup>-/-</sup> embryos never develop neurotrophin dependence *in vitro*, regardless of NGF exposure and withdrawal paradigms (Vogel et al., 1995). This may reflect the fact that few, if any, SCG neurons isolated at E13.5 (the latest stage to which *Nf1*<sup>-/-</sup> mouse embryos survive) have extended axons towards peripheral targets, and certainly none have contacted their targets *in vivo*. To determine whether NGF dependence could be induced in sympathetic neurons by exposure to the neurotrophin, we subjected E12.5 *Nf1*<sup>-/-</sup> and *Nf1*<sup>+/-</sup> SCG neurons to the NGF withdrawal paradigm described above for DRG neurons. **Figure 3** shows that NGF dependence fails to develop in 60% of *Nf1*<sup>+/-</sup> SCG neurons isolated at this early stage, and that very few of these cells can be rescued by depolarization. In contrast, NGF withdrawal has very little effect on the survival of SCG neurons isolated from *Nf1*<sup>-/-</sup> littermates (**Figure 3**). *These results raise the possibility that the role of neurofibromin in regulating acquisition of neurotrophin dependence may be very different for sensory and sympathetic neurons.* Within the next year, we plan to complete experiments, proposed in the last report, involving: pre-target contact DRG neurons (E11.5), “pulses” of NGF exposure (DRG and SCG), survival responses to suboptimal NGF doses (DRG and SCG), and prolonged (7-10 days) NGF exposure (SCG). In addition, we may include immunocytochemical analyses of c-jun kinase and c-jun phosphorylation in response to NGF withdrawal, for E13 *Nf1*<sup>-/-</sup> and *Nf1*<sup>+/-</sup> sensory and sympathetic neurons (see Task 7).

**Figure 3. NGF Withdrawal  
Survival of E12.5 SCG Neurons**



**Figure 3.** Dissociated cultures of E12.5 SCG neurons were maintained in 5ng/ml NGF in serum-free medium for 48 hours, at which point NGF was withdrawn. NGF, KCl, or no factors were added to the deprived cultures. Survival is expressed as a percentage of the initial cohort of neurons counted for each culture at 24 hours.

**Task 7. Characterize the susceptibility of neurofibromin-deficient neurons to ceramide-mediated apoptosis.**

In cultured cortical neurons, increased ceramide levels, generated by cleavage of sphingomyelin, activate c-jun and p38 kinases following the induction of apoptosis (Williams-Morawek et al., 2003). Addition of cell-permeable forms of ceramide induces apoptosis in cultured neurons and neuronal cell lines, and this apoptotic response can be blocked by overexpression of PI3 kinase or constitutively activated Akt (Zhou et al., 1998; Goswami et al., 1999). We proposed that *Nf1*<sup>-/-</sup> sensory neurons might be resistant to ceramide-induced apoptosis, due to constitutive activation of the PI3 kinase/Akt survival signaling pathway. In contrast to our predictions, we found that ceramide can induce apoptosis in neurofibromin-deficient neurons, even in the presence of NGF. In a recent paper on ceramide-induced apoptosis in cultured cortical neurons, both c-jun kinase (JNK) and c-jun phosphorylation were examined immunocytochemically (Williams-Morawek et al., 2003). We will continue to examine the time course of phosphorylation of these proteins in E13 *Nf1*<sup>-/-</sup> and <sup>+/+</sup> DRG and SCG neurons, following C2-ceramide exposure, over the next year, and plan to combine the results obtained from Tasks 4 and 7 in one manuscript. Although our completed and continuing experiments do not involve manipulations of gene expression in developing neurons, they do identify possible apoptotic signaling pathways, and begin to address some of the mechanisms for acquisition of neurotrophin dependence.

**KEY RESEARCH ACCOMPLISHMENTS**

- Completed NGF withdrawal and KCl rescue experiments for E13, E15, and E17 SCG sympathetic neurons, isolated from *Nf1*<sup>+/+</sup> and <sup>+/+</sup> mouse embryos, to complement previous results with DRG sensory neurons (Task 1).

- Completed experiments to examine possible "synergy" between neurotrophin- and activity-mediated survival signaling for *Nf1*<sup>+/-</sup> and <sup>+/+</sup> DRG and SCG neurons for 3 different embryonic ages (Task 3).
- Characterized effects of NGF withdrawal on E12.5 *Nf1*<sup>-/-</sup> SCG sympathetic neurons, to complement previous results with DRG sensory neurons. Began to examine effects of duration and timing of NGF exposure, and of target contact, on sensitivity to NGF withdrawal (Task 4).
- Continued to characterize apoptotic effects of C2-ceramide on E12.5/E13 *Nf1*<sup>-/-</sup> and <sup>+/-</sup> DRG and SCG neurons; examined expression of phosphorylated jun kinase and c-jun proteins in neurons (Task 7)

## REPORTABLE OUTCOMES

### Funding Obtained and Pending

- San Antonio Cancer Institute: " *Nf1* and Mrg15 Regulation of Tumorigenesis and Neural Cell Proliferation"
- Pending, DoD NFRP: "*Nf1* Expression: Computational Analyses and Experimental Verification of Putative Cis-Regulatory Sequences"
- Pending, DoD NFRP: "Variable Expressivity in NF1: Using Mouse Models to Identify Contributions of Genomic Instability"

### Employment and Training Opportunities

- Rene Garza, Research Assistant
- Robert Hudson III, Senior Research Assistant

### Manuscripts

- Brannan, C.I., and Vogel, K.S. (in preparation) Reduction in neurofibromin expression modulates the response to neurotrophin- and activity-mediated survival signaling in sensory and sympathetic neurons.
- Vogel, K.S. (in preparation) Loss of neurofibromin alters the development of neurotrophin dependence: Effects of NGF exposure

## CONCLUSIONS

Importance and Implications. Our results to date support the emerging idea that neurofibromin expression and *Nf1* haploinsufficiency influence the behavior of both peripheral and central neurons. Loss of neurofibromin, with the resulting abnormalities in Ras and PI3 kinase signaling, has profound effects on the neurotrophin dependence and sensitivity of embryonic sensory neurons (Vogel et al., 1995; Klesse and Parada, 1998; Vogel et al., 2000). Behavioral experiments with *Nf1*<sup>+/-</sup> and *exon23a*<sup>-/-</sup> mice indicate that neurofibromin function in CNS neurons modulates learning and memory (Silva et al., 1997; Costa et al., 2001). We have incorporated the reviewer's suggestion to utilize sympathetic neurons (from the superior cervical ganglion, SCG) in the experiments outlined in Tasks 1, 3, and 4. We have shown that *Nf1* haploinsufficiency affects both neurotrophin- and activity-mediated survival signaling for sensory and sympathetic neurons, at least by embryonic day 15 in the mouse; the differences between *Nf1*<sup>+/-</sup> and <sup>+/+</sup> neurons appear to become more significant with age. Our results may have implications for two areas: 1) the pathogenesis of learning disabilities in children with NF1, and 2) therapeutic strategies or targets for prolonging neuron survival, or for increasing neuronal response to protective agents, following injury or damage.

Neurotrophin withdrawal experiments involving E12.5 *Nf1*<sup>-/-</sup> and <sup>+/-</sup> SCG neurons revealed a difference between sympathetic and sensory neurons at this early stage. Whereas a proportion of neurofibromin-deficient DRG neurons die after NGF withdrawal (given prior NGF exposure), none of the NGF-deprived SCG neurons underwent apoptosis, even after a period of several days. Experiments with younger DRG



neurons and with differential exposure to NGF, in addition to analyses of JNK and c-jun phosphorylation in SCG and DRG neurons, should begin to reveal the basis for these differences in the response of *Nf1*<sup>-/-</sup> neurons.

**"So What" Section.** The learning disabilities associated with NF1 constitute a highly variable phenotype, and in addition represent a controversial topic of research and clinical interpretations. Using mice that harbor targeted mutations in *Nf1*, Silva and colleagues have demonstrated that aberrant or reduced regulation of Ras signaling by neurofibromin may contribute to certain aspects of the spatial learning disorder (Silva et al., 1997; Costa et al., 2001). More recently, these researchers have proposed that the excessive Ras activity in *Nf1*<sup>+/-</sup> neurons leads to increased GABA-mediated inhibition and defects in long-term potentiation (Costa et al., 2002). Our results in Tasks 1 and 3, for both sensory and sympathetic neurons, are consistent with the interpretation that *Nf1*<sup>+/-</sup> neurons may respond aberrantly to electrochemical (ion gradients) and neurotrophin stimuli, which could potentially affect neuronal function and synaptic transmission. To relate these *in vitro* results to the complex issue of NF1-related learning disorders, it may be of interest to use computer modeling to characterize possible consequences of additional neurons (particularly inhibitory ones) in a circuit, or of aberrant signaling by neurons within a given circuit. Our results with *Nf1* haploinsufficient neurons also point to neurofibromin as a possible therapeutic target following neuronal injury; reduction in neurofibromin activity may prolong neuron survival, or enhance the response to protective agents.

Both neurotrophin signaling and activity-mediated processes are required to achieve correct target innervation patterns and synaptic plasticity in the peripheral nervous system (Davies, 2003). For many types of embryonic neurons, activation of the intracellular signaling pathways required for these processes often correlates with the timing of target contact. To date, our experiments indicate possible differences in the role of neurofibromin in acquisition of neurotrophin dependence, for embryonic sensory and sympathetic neurons. Both molecular mechanisms and the importance of target contact can be addressed readily in our *in vitro* system, and should contribute to our understanding of how neurotrophin signaling pathways are established during development. Recently, the transcription factor Brn3a (Ma et al., 2003) and cofactor HIPK2 (Wiggins et al., 2004) have been identified as important regulators of pro-survival gene expression in developing sensory neurons; overexpression of HIPK2 in cultured neonatal trigeminal neurons induces apoptosis (Wiggins et al., 2004).

Recent experiments with cancer cells have led to the concept of "oncogene addiction" (reviewed by Jonkers and Berns, 2004). For example, Buzzai and colleagues (2005) reported that cancer cells with activated Akt have increased glucose uptake and metabolism, and are susceptible to apoptosis following glucose withdrawal because they can no longer metabolize nonglycolytic bioenergetic substrates. Loss of, or reduction in, neurofibromin function leads to activation of Ras signaling in both cancer cells and in postmitotic neurons, thus potentially creating a type of "oncogene addiction". Moreover, aberrant activation of Akt and other signaling pathways in *Nf1*<sup>+/-</sup> and *Nf1*<sup>-/-</sup> neurons may lead to defects in metabolism and synaptic transmission, and contribute to the learning disorders associated with NF1.

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## APPENDICES

Not Applicable